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14. ABSTRACT
This project focuses on the potential role of the calcium-selective ion channel, CaT1, in prostate cancer progression. Although the physiological role of CaT1 is still poorly understood, it may be a "store-operated" calcium pore, a type of channel that is known to play a role in cell growth and survival regulation. The Aims of this project are to (1) Develop specific antibodies against CaT1 and characterize its expression pattern(s) in human prostate cancer tissues; and to (2) Determine the role of CaT1 in cell growth and survival regulation in human prostate cancer cells. We have successfully completed most of the objectives in Aim (Task) 1. During the first funding year, we developed and characterized a CaT1 antibody suitable for highly specific detection of the CaT1 protein in tissues. We used this novel reagent to characterize the expression patterns of CaT1 protein in mouse and human; we discovered that CaT1 is expressed by a number of exocrine organs; and we discovered that CaT1 is over-expressed in prostate, breast, thyroid, colon and ovarian carcinomas. These findings indicate that CaT1 is likely to serve as a component of transcellular calcium transport mechanisms in many tissues and epithelial cancers.

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INTRODUCTION

This project focuses on the ion channel protein, TRPV6 (CaT1), originally described in intestinal cells but now known to be expressed in a number of tissues. Gene expression analysis indicates that TRPV6 is present at high levels in the prostate (10-50-fold higher levels than in most other organs). The physiological role of TRPV6 is unknown; however, it has been functionally characterized by cloning, gene transfer and electrophysiological measurements in cell lines and frog eggs. These experiments have revealed that TRPV6 is a calcium-selective channel of the plasma membrane. It appears to function as a calcium entry pore, whereby ionic calcium enters the cell in response to defined triggers. TRPV6 is a not the type of calcium channel that is "voltage-gated", such as those present in electrically excitable cells. Evidence indicates, however, that TRPV6 may be a type of "store-operated " calcium pore. These types of channels are known to play a role in cell growth and survival regulation.

Our laboratory demonstrated in a published report that expression of the TRPV6 gene is associated with human prostate cancer progression (Peng et al., 2001). We have also shown in experiments with prostate cancer cell lines that TRPV6 expression increases under conditions of androgen deprivation. These findings, along with the known properties of TRPV6/store-operated calcium channels, indicate that this protein may play an important functional role in regulating prostate cancer cell survival, including under conditions of androgen suppression. The link between a calcium entry channel and androgen-independent prostate cancer is novel, and potentially related to mechanisms of progression of hormone-insensitive cancer and to epidemiological data pointing to increased risk of prostate cancer from consumption of dairy products.

In this Idea Development Award, we proposed two primary objectives: (1) develop new probes (antibodies) to study the TRPV6 protein in tissues; and (2) use molecular and cell biological methods to explore the functional role of TRPV6 in prostate cancer cells.

BODY

Task 1. Develop specific antibodies against TRPV6 and characterize its expression pattern(s) in human prostate cancer tissues.

Task 1 was largely accomplished. Details of the development and use of TRPV6 antibodies to study TRPV6 expression in prostate and other tissues in human and mouse were reported to the DoD in our year 1 progress report. These studies resulted in the publication of an original research article in a major journal (Zhuang et al., 2002).

Task 2. Use molecular and cell biological methods to explore the functional role of CaT1 in prostate cancer cells.

Our published studies have linked TRPV6 to aggressive (hormone-refractory) prostate cancer. Other recent studies from our group have demonstrated that prostate

cancer cells employ a cholesterol-rich plasma membrane (lipid raft) compartment to transmit cell growth and survival signals (Zhuang et al. 2002; Kim et al 2004). Consequently, we have carried out a series of experiments to assess whether signal transduction mechanisms employing TRPV6 use lipid raft microdomains as part of the signaling process. In the past year we have accumulated substantial unpublished data to indicate that this hypothesis may be true and that TRPV6 may play a major role in proliferative and survival signals relevant to prostate cancer progression. Attempting to identify such a mechanism was a major goal of the original grant proposal.

Finding 1: CaT1 localizes to prostate cancer cell lipid rafts

Lipid rafts are cholesterol- and sphingolipid-enriched microdomains in cell membranes, which have been proposed by us and others to serve as platforms for various cell signaling and transport processes. To examine the subcellular localization of TRPV6 and explore its potential functional relationship with lipid raft microdomains in prostate cancer cells, we isolated lipid raft membranes from LNCaP prostate cancer cells by flotation in sucrose gradients and probed gradient fractions with antibodies for known raft proteins, such as Fyn and Giα2 (Figure 1). (Identification of TRPV6 protein in these experiments was possible because we developed a highly specific anti-TRPV6 antibody from the studies funded by this grant). We found that most of the TRPV6 protein floated in sucrose gradients and co-localized with known raft marker proteins, indicating that TRPV6 is present predominantly in lipid rafts in LNCaP cells. The observation that TRPV6 localizes to lipid raft membranes implies a functional dependency between this cholesterol-rich membrane compartment and TRPV6-mediated calcium influx.

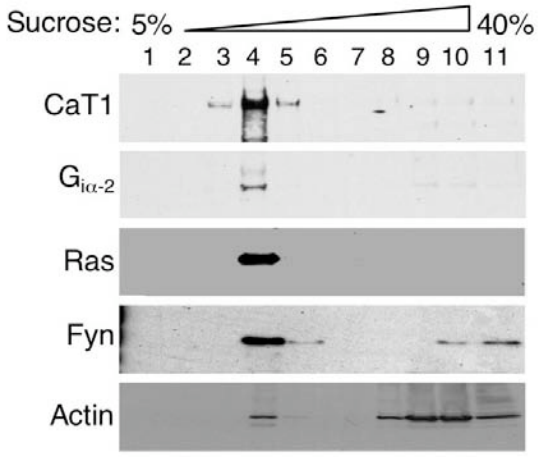

Figure 1. ***CaT1/TRPV6 localizes to light buoyant density lipid raft fractions in sucrose gradients.*** LNCaP cells were lysed using a detergent-free method and prepared for sucrose density ultracentrifugation. CaT1 localizes in the gradients with known lipid raft markers, including Giα2 and the Src-like kinase, Fyn.

Finding 2: Regulated calcium influx is potentiated by cholesterol addition to cell membranes

To further investigate the novel possibility that calcium entry is regulated by a lipid raft-dependent mechanism, we manipulated a key raft component, cholesterol, in calcium influx experiments. In experiments where store-operated calcium influx was triggered by thapsigargin (an inhibitor of the endoplasmic reticulum calcium pump), addition of exogenous cholesterol to cell membranes dramatically potentiated calcium influx (Figure 2). This effect was inhibited by the cholesterol-binding reagents, methyl-β-cyclodextrin and filipin. Further, the inhibitory effect of the calcium-binding compounds was completely reversed by restoration of membrane cholesterol levels by addition of exogenous cholesterol. These experiments are consistent with the data shown in

Figure 1, where TRPV6 was found to localize to lipid raft microdomains, and they suggest that regulated influx of calcium ions in LNCaP cells is mediated by cholesterol-rich lipid raft microdomains.

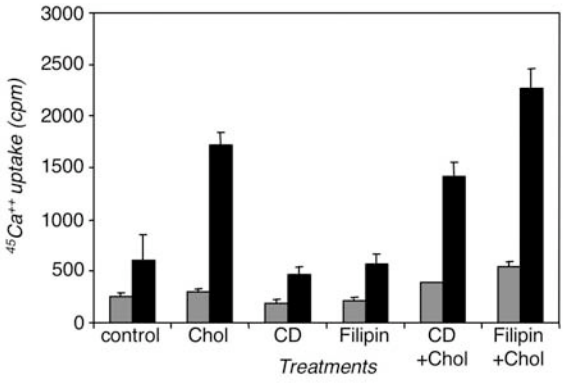

Figure 2. ***Cholesterol potentiates store-operated calcium influx in LNCaP cells.*** Experimental groups were as follows: (1) control: (+/- thapsigargin treatment; (black bars are thapsigargin-treated in all conditions); (2) cholesterol pre-treament for 1 h (Chol); (3) 20 μM cyclodextrin treated for 1 h after cholesterol pretreatment (CD); (4) 2mg/ml filipin treatment for 1 h after cholesterol pre-treatment; (5) cholesterol reloaded after treatment with CD; (6) cholesterol reloaded after treatment with filipin. After the above manipulations, cells were harvested and $3x10^5$ cells were used for thapsigargin-induced calcium uptake assay according to Peng et al. J. Biol. Chem. 274:22739-22746, 1999.

Finding 3: Regulated calcium influx is dependent on intact lipid rafts

Calcitonin is a calciferic hormone that is secreted by parafollicular cells of the thyroid gland. Its function is to maintain calcium homeostasis by several mechanisms, including inhibiting osteoclast activity in bone, decreasing absorption of calcium ions from the small intestine, and decreasing calcium excretion from the kidney. Its possible role as a trophic factor involved in prostate cancer growth is unknown. We demonstrated that calcitonin upregulates TRPV6 levels in a dose-dependent manner in the LNCaP cell line (Figure 3), providing a link between this hormone and a calcium-dependent signaling mechanism in human prostate cancer cells. Interestingly, calcitonin also activated signaling from the epidermal growth factor receptor family members EGFR/ErbB1 and ErbB2. These data provide evidence for a link between calcium regulation and well-known cell proliferation and survival pathways in prostate and other cancer cells.

Finding 4 : LNCaP cells express high levels of CaT1/TRPV6 and high calcium influx

Consistent with our previous demonstration that prostate cancer cells in human tumor tissue express high levels of CaT1/TRPV6, we have demonstrated that LNCaP cells, a continuous cell line used widely as a prostate cancer model, express high levels of the protein and high calcium influx in comparison to HEK293 cells (Figure 4). These data provide a basis for use of the LNCaP cell for functional studies of the role of CaT1/TRPV6 in a human prostate cancer cell context.

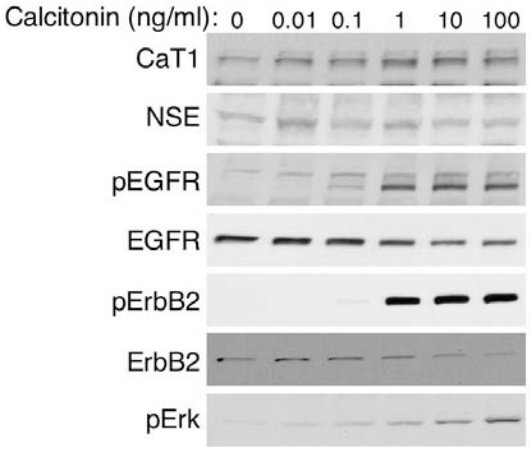

Figure 3. ***Calcitonin induces CaT1/TRPV6 expression and activates EGFR family receptor tyrosine kinases in LNCaP cells.*** pEGFR and pErbB2 indicate phosphorylated forms of the two receptors detected by phospho-site-specific western blot. pERK refers to the phosphorylated form of the ERK mitogen activated protein kinase. NSE=neuron-specific enolase.

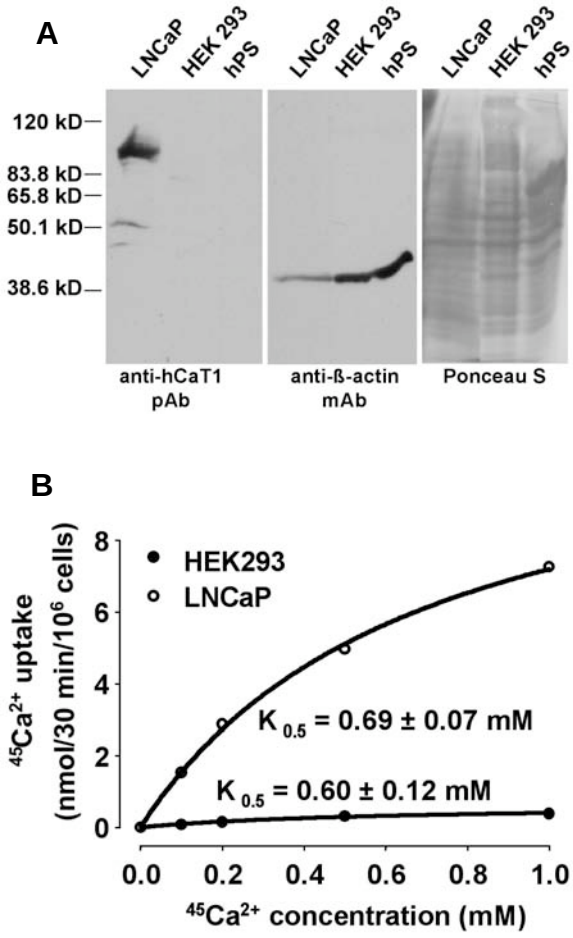

Figure 4. ***LNCaP cells express high levels of CaT1/TRPV6 and high calcium influx.*** **A .** Total cell lysates of LNCaP PCa cells, human embryonic kidney (HEK293) cells, and normal human prostate stroma cells (hPS) were subjected to immunoblot using anti-human CaT1 (hCaT1) polyclonal antibody (pAb) CH2747 (1:2000) raised in chicken. The same blot was also probed with anti-β-actin monoclonal antibody (mAb). Ponceau S staining of the blot demonstrates equal protein loading. **B.** Concentration-dependent $^{45}Ca^{2+}$ uptake in LNCaP in comparison to HEK 293 cells.

Finding 5 : Stable knock-down of CaT1/TRPV6 in LNCaP cells

Having demonstrated that LNCaP cells are suitable for the functional study of CaT1/TRPV6, we used an RNA interference (RNAi) approach to stably knock-down expression of CaT1/TRPV6 in this cell line. LNCaP cells with dramatically lowered CaT1/TRPV6 have been generated using this method (Figure 5).

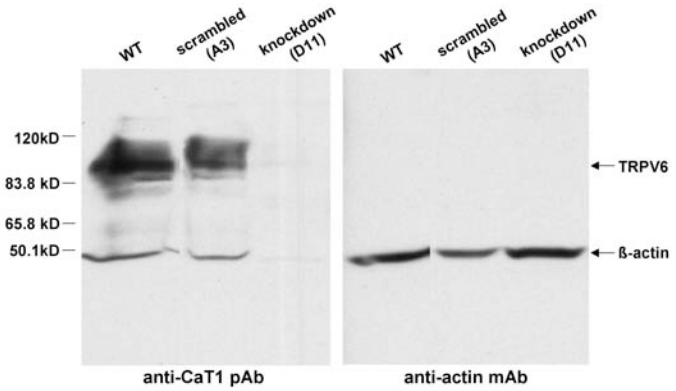

Figure 5. ***Down-regulation of CaT1/TRPV6 in LNCaP cells by RNA interference.*** A 21-nt double-stranded RNA with the sequence AAC CUG CUG CAG CAG AAG AGG was was assembled into a viral vector, pSUPER.retro.puro. Viruses were generated as described online at *www.oligoengine.com.*After viral infection, cells were screened in medium containing puromycin (3 μg/ml) for 3-6 w. Control and stable CaT1 knockdown cells were subjected to immunoblotting with anti-hCaT1 antibody. The same blot was probed with anti-β-actin as internal control.

Finding 6 : LNCaP cells with down-regulated CaT1/TRPV6 exhibit an altered phenotype

The original hypothesis in our research proposal was that CaT1/TRPV6 is a direct mediator of the biological behavior of prostate cancer cells. To test this possibility, we assessed (1) cell growth in the presence of serum and (2) motile behavior in response to SDF-1, a chemokine that mediates cell motility through the action of the CXCR4 receptor. Our results indicate that both cell behaviors were suppressed in LNCaP cells where CaT1/TRPV6 was stably down-regulated in comparison to cells transfected with scrambled DNA (Figure 6A,B).

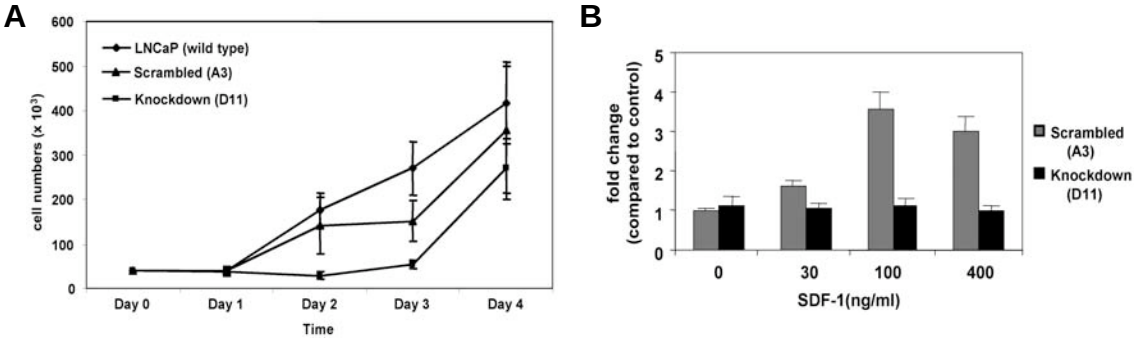

Figure 6. Biological effects of CaT1/TRPV6 lowering in LNCaP cells. **A.** Cells (4×10^4/well) were seeded in 24-well plates. At daily intervals, cells were trypsinized and counted. Growth curves of the three cell populations are shown as mean ± SD in triplicate wells. **B.** Chemotaxis was assayed using Falcon Transwells (24-well; BD PharMingen). 5×10^5 cells were added to the upper chamber, 0.5 ml of medium alone or media containing SDF-1 were added to the lower chamber. After overnight incubation, migrated cells on the lower surface of the filter were stained with crystal violet. The number of migrated cells in 10 medium-power fields (x20) was counted. The mean ± SD in triplicate experiments was shown.

Finding 7. CaT1/TRPV6 interacts with the actin-binding protein abLIM

In order to gain insight into the manner in which calcium influx is regulated by cholesterol in LNCaP cells, and the apparent role of CaT1/TRPV6 in tumor cell growth and chemotaxis, yeast two hybrid analysis was performed to identify possible cooperating proteins. These studies identified the actin-binding protein, abLIM (UNC-115), as a potentially physiologically relevant partner of CaT1/TRPV6. Two hybrid data demonstrating direct interaction between CaT1/TRPV6 and abLIM in yeast were verified in mammalian tissues (pancreas and duodenum, which have been demonstrated to express high levels of CaT1/TRPV6) by co-immunoprecipitation (Figure 7).

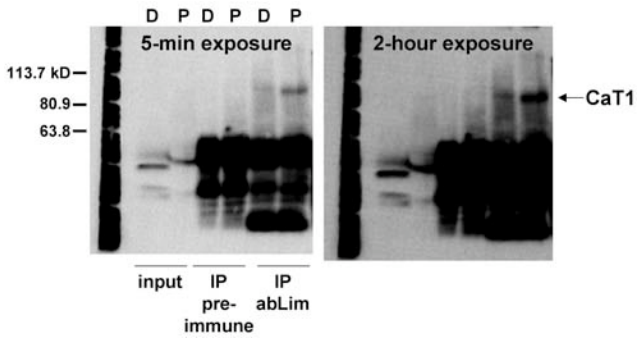

Figure 7. CaT1/TRPV6 interact in vivo. Mouse duodenum (D) and pancreas (P) tissues were minced and then lysed in RIPA buffer. Immunoprecipitation was performed using rabbit anti-human abLIM antiserum and protein A beads. Beads were boiled in SDS loading buffer, resolved in a 4-20% gradient gel and transferred to nitrocellulose membrane. Western blot was performed with anti-human CaT1/TRPV6 antibody raised in rabbit (1:500 dilution).

Finding 8. CaT1/TRPV6 and abLIM co-localize in mouse tissues

Additional evidence for a physiological interaction between CaT1/TRPV6 and abLIM by immunostaining of both proteins in mouse tissues. Figure 8 demonstrates that CaT1/TRPV6 and abLIM co-localize in mouse duodenum and pancreas (Figure 8A) and prostate (Figure 8B).

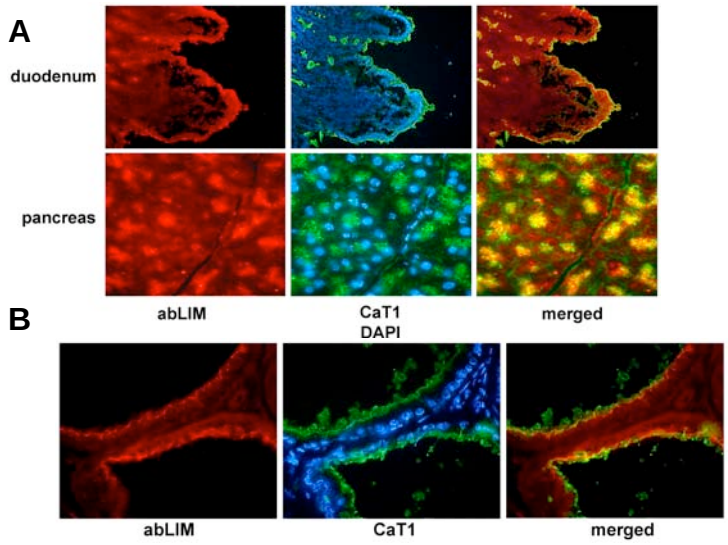

Figure 8. **Co-localization of CaT1/TRPV6 and abLIM in mouse tissues.** Immunofluorescence staining was performed using antibodies that specifically detect murine CaT1/TRPV6 and abLIM proteins. **A.** Duodenum and pancreas. **B.** Prostate.

Finding 9. Development of an inducible CaT1/TRPV6 expression system

In order to study CaT1/TRPV6 functionally, we have been attempting to engineer several continuous cell lines to generate cellular systems where we can induce conditional expression of the protein. To date, we have succeeded in constructing a CaT1/TRPV6-inducible cell line using HEK 293 cells, which do not express detectable levels of endogenous CaT1/TRPV6. In Figure 9, we demonstrate the induction of an EGFR-CaT1/TRPV6 fusion protein and the appearance of a CaT1/TRPV6 current in this cell system.

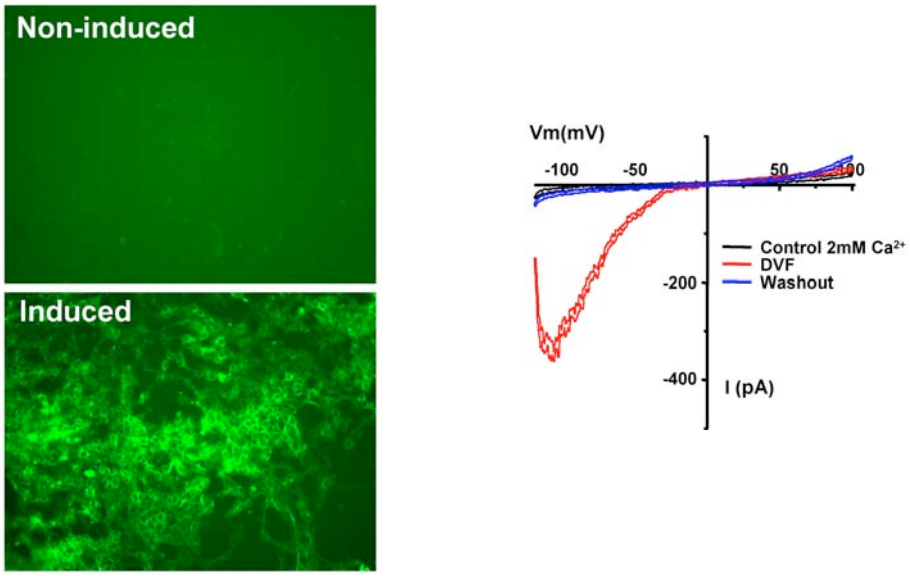

Figure 9. A conditional system for CaT1/TRPV6 expression. HEK 293 cells were transfected with an expression vector containing a construct encoding an EGFP-CaT1/TRPV6 fusion protein within an ecdysone gene expression cassette. Resultant antibiotic-resistant cell populations were isolated and characterized. In the figure we demonstrate induction of expression of the fusion protein. **A**. HEK 293 TRPV6-EGFP cells were treated with 5μM ponasterone for 20 h. Green fluorescence, originating from the EGFP moiety, was observed in live cells under divalent ion-free conditions. Control cells showed negligible green. **B.** The characteristic CaT1/TRPV6 current was detected by patch clamp.

REPORTABLE OUTCOMES

To date, **seven** full-length articles have been published as a result of funding from this grant:

Zhuang, L., Peng, J-B., Tou, L., Takanaga, H., Adam, R.M., Hediger, M.A., and **Freeman, M.R**. (2002) Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. Laboratory Investigation 82:1755-1764.

Freeman, M.R., and Solomon, K.R. (2004) Cholesterol and prostate cancer. Journal of Cellular Biochemistry 91:54-69.

Cinar, B., Yeung, F., Konaka, H., Mayo, M.W., **Freeman, M.R**., Zhau, H.E., and Chung, L.W.K. (2004) Identification of a negative regulatory cis-element in the enhancer core region of the Prostate Specific Antigen (PSA) promoter: Implications for intersection of androgen receptor and NF-kappaB signaling in prostate cancer cells. Biochemical Journal 379:421-431.

Kim, J., Adam, R.M., Solomon, K.R., and **Freeman, M.R.** (2004) Involvement of cholesterol-rich lipid rafts in interleukin-6-induced neuroendocrine differentiation of LNCaP prostate cancer cells. Endocrinology 145:613-619.

Zhuang, L., Kim, J., Adam, R.M., Solomon, K.R., and **Freeman, M.R**. (2005) Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. Journal of Clinical Investigation 115:959-968.

Cinar, B., De Benedetti, A., and **Freeman, M.R**. (2005) Post-transcriptional regulation of the androgen receptor by the mammalian target of rapamycin. Cancer Research 65:2547-2553.

Freeman, M.R., Cinar, B., and Lu, M.L. (2005) Membrane rafts as potential sites of non-genomic hormonal signaling in prostate cancer. Trends in Endocrinology and Metabolism (In press).

CONCLUSIONS

We believe that we have now made the first link between regulated, store-operated calcium influx and cholesterol- and growth factor-dependent regulatory mechanisms in prostate cancer. These results relate directly to several hypotheses we sought to test in the original proposal, including the possible influence of diet (e.g., calcium, cholesterol) on regulatory mechanisms involved in prostate cancer progression. We anticipate that at least one additional original report will be published as a result of funding by this grant. We also believe we have assembled sufficient preliminary data for submission of a competitive NIH R01 grant application on the role of CaT1/TRPV6 on calcium-dependent regulation of prostate cancer growth and survival mechanisms.

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Kim, J., Adam, R.M., Solomon, K.R., and **Freeman, M.R.** (2004) Involvement of cholesterol-rich lipid rafts in interleukin-6-induced neuroendocrine differentiation of LNCaP prostate cancer cells. Endocrinology 145:613-619.

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Zhuang, L., Peng, J-B., Tou, L., Takanaga, H., Adam, R.M., Hediger, M.A., and Freeman, M.R. (2002) Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. Lab. Invest. 82:1755-1764.